TRANSLATOR'S DECLARATION

I, John F. Moloney, Bsc., MIL., CChem., MRSC., translator to Taylor and Meyer of 20 Kingsmead Road, London, SW2 3JD, Great Britain, verify that I know well both the German and the English language, that I have prepared the attached English translation of 12 pages of a German Patent application in the German language with the title:

Verwendung der Acetylaminosäureracemase aus Amycolatopsis orientalis zur Racemisierung von Carbamoylaminosäuren

identified by the code number 000337 AM at the upper left of each page and corresponding to client/matter number of the law firm of

and that the attached English translation of this document is a true and correct translation of the document attached thereto to the best of my knowledge and belief.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of this document.

By: J. F. Moloney

Date: 31st July 2003

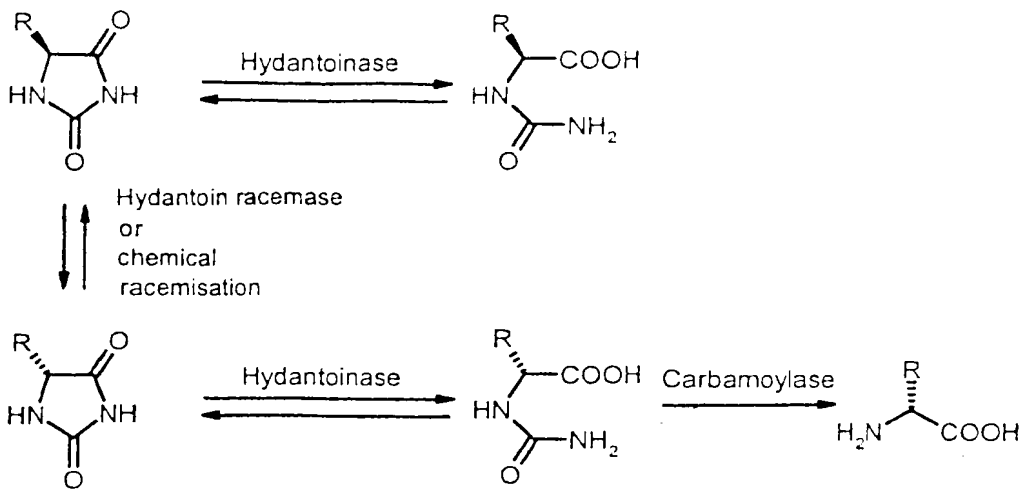
Use of acetylamino acid racemase from Amycolatopsis orientalis for racemisation of carbamoylamino acids

The present invention relates to the use of an N-acetyl-amino acid racemase (AAR) in a process for the racemisation of N-carbamoylamino acids.

Optically pure amino acids are important starting materials for chemical synthesis and for parenteral nutrition. Many possibilities of preparing optically pure amino acids are known to the skilled person. Enzymatic processes, i.a. are suitable in this respect since, on the one hand, they operate catalytically and on the other hand permit the preparation of the amino acids with very high enantiomer enrichment.

A known enzymatic process starts from racemic hydantoins which are transformed to N-carbamoyl-protected amino acids by means of hydantoinases. These are then converted by carbamoylases to the amino acids.
The separation of the racemates occurring in this reaction sequence takes place preferably on the basis of the N-carbamoyl-protected amino acids because both L and D-selective carbamoylases are available (Park et al., Biotechnol. Prog. 2000, 16, 564-570; May et al., Nat Biotechnol. 2000, 18, 317-20; Pietzsch et al., J. Chromatogr. B Biomed. Sci. Appl. 2000, 737, 179-86; Chao et al., Biotechnol. Prog. 1999, 15, 603-7; Wilms et al., J. Biotechnol. 1999, 68, 101-13; Batisse et al., Appl. Environ. Microbiol. 1997, 63, 769-6; Buson et al., FEMS Microbiol. Lett. 1996, 145, 55-62).
In order to guarantee complete conversion of the hydantoins used to optically pure amino acids, the necessary racemisation has taken place hitherto on the basis of hydantoins by chemical or enzymatic means (EP 745678; EP 542098; scheme 1).

Scheme 1:



N-acetylamino acid racemases (AARs) from Streptomyces atratus Y-53 (Tokuyama et al., Appl. Microbiol. Biotechnol. 1994, 40, 835-840) and Amycolatopis sp. TS-1-60 (Tokuyama et al., Appl. Microbiol. Biotechnol. 1995a, 42, 853-859) and Amycolatopsis orientalis sp. lurida (DE19935268) are known. TS-1-60, however, is found to have a very low activity in the case of N-carbamoyl-protected amino acids. Moreover, this enzyme has the disadvantage of a very high metal ion dependence, which appears to be a drawback for the use of this enzyme in an industrial-scale process.

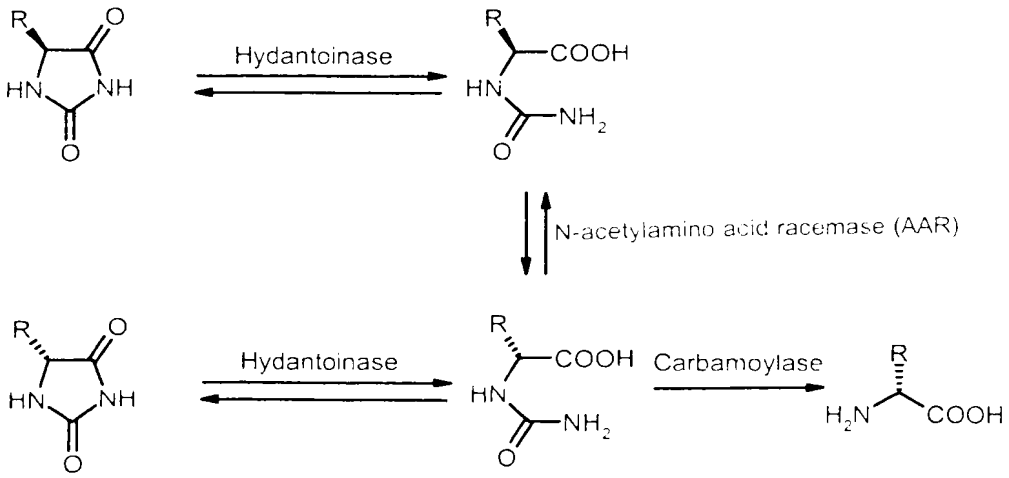
The object of the present invention was, therefore, to show the use of an N-acetylamino acid racemase for the improved racemisation of N-carbamoylamino acids compared with the prior art. The intention was that this racemase might be used advantageously on an industrial scale in a process for the preparation of optically pure amino acid starting from racemic hydantoins.

The object is achieved by the use of the AAR according to claim 1. Claims 2 and 3 relate to preferred embodiments of the racemisation process according to the invention.

Due to the fact that an N-acetylamino acid racemase (AAR) from Amycolatopsis orientalis subspecies lurida (seq. 2) is used in a process for the racemisation of N-carbamoylamino acids, and in view of the surprisingly high activity of the AAR used according to the invention compared with TS-1-60 in terms of the racemisation of N-carbamoylamino acids, it is possible to achieve an equilibrium of enantiomers of N-carbamoyl-protected amino acids in an improved process.

This is particularly advantageous in that it is thus possible to establish a further enzymatic step in a process for the preparation of optically pure amino acids which is based on hydantoins (scheme 2).

Scheme 2:



In contrast to the enzymatic processes known from the literature and which proceed by way of enzymatic or optionally stressing chemical racemisation of hydantoins (scheme 1), a further advantageous possibility of generating optically pure amino acids from racemic hydantoins has thus been created.

The variant of AAR from Amycolatopsis o. sp. lurida prepared by recombinant technology according to DE19935268 is preferably used for the racemisation process. It is

known from DE19935268 that this exhibits relatively little heavy metal ion dependence (particularly with regard to cobalt ions) and has low amino acid inhibition. The generation thereof as a recombinant enzyme is also explained therein.

The process according to the invention, as has been mentioned, is used advantageously in an overall process for the preparation of enantiomerically enriched amino acids or derivatives thereof starting from hydantoins or N-carbamoylamino acids. In the case of hydantoins, it is preferable to proceed in such a manner that racemic hydantoins are cleaved by hydantoinases into the corresponding racemic N-carbamoylamino acids and these are then converted by L- or D-specific carbamoylases into the optically active L- or D-amino acids. To ensure that no enrichment of the unconverted enantiomer of an N-carbamoylamino acid takes place in the reaction mixture, the enantiomers of the N-carbamoylamino acids are brought into equilibrium by the addition of the AAR according to the invention and it is thus likewise possible to convert the racemic hydantoin wholly to optically pure amino acids.

This process takes place preferably in an enzyne-membrane reactor (DE 199 10 691.6).

The enzymes mentioned may be used together or successively in the free form as homogeneously purified compounds or as enzymes prepared by recombinant technology. Moreover, the enzymes may also be used as a constituent of a guest organism (whole-cell catalyst as in US09/407062)or in conjunction with the digested cell mass of the host organism. It is also possible to use the enzymes in the immobilised form (Bhavender P. Sharma, Lorraine F. Bailey and Ralph A. Messing, "Immobilisierte Biomaterialiern - Techniken und Anwendungen", Angew. Chem. 1982, 94, 836-852). Immobilisation takes place advantageously by freeze-

drying (Dordick et al. J. Am. Chem. Soc. 194, 116, 5009-5010; Okahata et al. Tetrahedron Lett. 1997, 38, 1971-1974; Adlercreutz et al. Biocatalysis 1992, 6, 291-305). Freeze-drying in the presence of surfactant substances such as Aerosol OT or polyvinylpyrrolidone or polyethylene glycol (PEG) or Brij 52 (diethylene glycol monocetyl ether) (Goto et al. Biotechnol. Techniques 1997, 11, 375-378) is more particularly preferred.

The microorganism Amycolatopsis orientalis subsp. lurida is deposited with the German Collection for Microorganisms under number DSM43134.

The term AAR within the context of the invention means both the native enzyme and the enzyme prepared by recombinant technology.

The term enantiomerically enriched denotes the presence of one enantiomer in the mixture with the other in a proportion of >50%.

The term amino acid within the context of the invention means a natural or non-naturally occurring α-amino acid, i.e., the radical situated on the α-C-atom of the α-amino acid may be derived from a natural amino acid as described in Beyer-Walter, Lehrbuch der organischen Chemie, S. Hirzel Verlag Stuttgart, 22nd edition, 1991, p.822f. or also from corresponding α-radicals of non-naturally occurring amino acids which are listed, e.g. in DE19903268.8.

SEQUENCE PROTOCOL

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Examples:

Detection of racemase activity of the recombinant AAR enzyme

The substrate spectrum of the N-acetylamino acid racemase from Amycolatopsis orientalis subsp. lurida was tested using the enzyme assay described below.

The assay was composed of the following:

Tris/HCl buffer	50 mM (pH 8.0)
Substrate	25 mM
Cobalt chloride	6 mM
AAR	approx 150 µg purified protein
Final volume	1 ml

Enantiomerically pure amino acid derivatives were used in the test and the formation of the corresponding racemate was monitored in the polarimeter (Perkin-Elmer 241). Incubation took place at 30°C (heated cell) for 3 to 12 hours. The measurements were taken at a wavelength λ = 365 nm.

Table 1: List of the substrates tested and of the corresponding specific activity of the AAR.

Substrate	Specific activity
N-Carbamoyl-D-Met	155 mU/mg
N-Carbamoyl-D-Phe	20 mU/mg
N-Carbamoyl-L-Abs	15 mU/mg
N-Carbamoyl-L-Leu	20 mU/mg
N-Carbamoyl-L-Met	118 mU/mg
N-Carbamoyl-L-Tyr	62 mU/mg
N-Carbamoyl-L-Val	20 mU/mg

The N-acyl amino acid racemase from A. TS-1-60 with N-carbamoyl-D-Met as substrate has an activity of 100 mU/mg. This specific activity is thus 35% lower than that of the racemase from A. orientalis subsp. lurida.

Patent claims:

1. Use of N-acetylamino acid racemases (AAR) from Amycolatopsis orientalis subspecies lurida in a process for the racemisation of N-carbamoylamino acids.

2. The use as claimed in claim 1 in a process for the preparation of enantiomerically enriched amino acids or derivatives thereof starting from hydantoins or N-carbamoylamino acids.

3. The use as claimed in one of the preceding claims, wherein
the process is carried out in an enzyme-membrane reactor.

Abstract:

The invention relates to the use of the N-acetylamino acid racemase from Amycolatopsis orientalis subspecies lurida for the racemisation of N-carbamoylamino acids.

This use permits the 100% preparation of optically pure amino acids starting from racemic hydantoins in an enzymatic overall process.